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ATTN: MCMR-CDT-N
3100 Ricketts Point Road

Aberdeen Proving Ground, MD 21010-5400

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ATTN: MCMR-CDZ-I
3100 Ricketts Point Road

Aberdeen Proving Ground, MD 21010-5400

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Evaluation of toosendanin as a botulinum neurotoxin antagonist

Michael Adler* and James D. Nicholson

Neurobehavioral Toxicology Branch,
Analytical Toxicology Division,
US Army Medical Research Institute of Chemical Defense,
3100 Ricketts Point Road, Aberdeen Proving Ground,
MD 21010-5400, USA
Fax: 410-436-2750
E-mail: Michael.adler2@us.army.mil
E-mail: jdn2916@gmail.com
*Corresponding author

Abstract: Toosendanin (TSN), a tetranortriterpenoid compound from the plant *Melia toosendan*, has been reported to protect rats and non-human primates from the lethal actions of botulinum neurotoxin A (BoNT/A). TSN was tested in isolated mouse phrenic nerve-hemidiaphragm preparations against BoNT serotypes A, B and E. When added prior to toxin, TSN was found to delay the onset of muscle paralysis with all 3 BoNT serotypes. TSN had little or no effect on BoNT-induced muscle paralysis when applied 30 min after BoNT. It is suggested that the antagonist activity of TSN may result from inhibition of BoNT translocation.

1   Introduction

Toosendanin (TSN) is a tetranortriterpenoid compound with a 4,4,8-trimethyl-17-furanylsteroid skeleton (Figure 1). TSN is one of the six identified triterpenoids that have been extracted from the fruit and bark of the Chinaberry tree, *Melia toosendan* (Wang and Wen, 1959; Shu and Liang, 1980; Tada et al., 1999). TSN has been used in traditional Chinese medicine for the treatment of intestinal parasites is currently under evaluation in contemporary Chinese medicine as an antimalarial, antiviral and antineoplastic agent (Shi and Wang, 2004). It is not clear whether TSN acts by a single mechanism, or whether multiple mechanisms are involved to account for its diverse actions. In isolated rat phrenic nerve-hemidiaphragm preparations, TSN was found to enhance acetylcholine (ACh) release in low concentrations, but at higher concentrations, TSN caused an initial facilitation followed by sustained depression of ACh release (Shih et al., 1980; Shih and Hsu, 1983). TSN was also found to block calcium-activated potassium currents in hippocampal neurons (Wang and Shi, 2001), to inhibit calcium currents in mouse *triangularis sterni* perineural membranes (Ding et al., 2001) and to depress voltage-dependent potassium currents in NG108-15 cells (Hu et al., 1997).

Figure 1   Molecular structure of TSN, a naturally occurring tetranortriterpenoid compound isolated from *Melia toosendan*
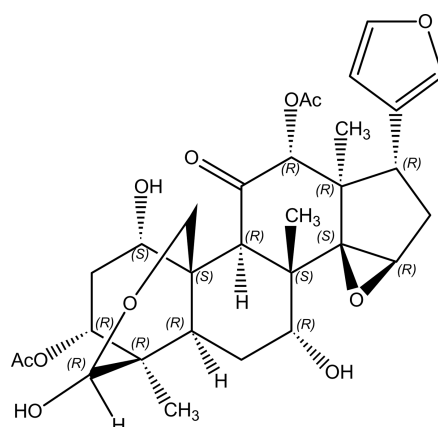
Owing to its enhancement of ACh release, TSN was examined for its ability to antagonise the actions of Botulinum Neurotoxin (BoNT), a highly potent bacterial neurotoxin that produces muscle paralysis by inhibiting ACh release (Simpson, 2004). In animal protection studies, TSN was found to antagonise the actions of BoNT in rats (Shih and Hsu, 1983) and in non-human primates (Jing et al., 1985). In the former study, rats were injected with 7 mg/kg TSN subcutaneously (s.c.) and sacrificed at various times after drug administration; their isolated diaphragms were tested for time-to-block of neurally elicited twitch tensions after addition of 2×10^4 mouse LD50 units/ml of BoNT/A (Shih and Hsu, 1983). Delays in the onset of BoNT-mediated muscle paralysis were observed for nearly a week after TSN administration. In the latter study, rhesus monkeys were exposed to a 1.7 LD50 dose of BoNT/A (s.c.) and TSN (0.9–1 mg/kg, s.c.) was administered 24 h after toxin challenge. Ten of 13 TSN-treated animals, but only 2 of 12 untreated animals, were able to survive BoNT challenge (Jing et al., 1985).

These studies are of interest since they demonstrate that protection by TSN persists for nearly a week after a single acute injection (Shih and Hsu, 1983) and that TSN is effective after BoNT exposure (Jing et al., 1985), a trait not observed with the majority of BoNT antagonists (Dickerson and Janda, 2006; Adler et al., 2007). The protective effect of TSN on BoNT-mediated toxicity was attributed to inhibition of potassium channels and a subsequent increase in calcium influx (Li and Sun, 1983; Zou et al., 1985), an action resembling that of the aminopyridine class of potassium blockers (Simpson, 1986; Adler et al., 1995, 1996, 2000).

In this study, we examined the interaction of TSN with BoNT/A, /B or /E on isolated mouse phrenic nerve-hemidiaphragm preparations to gain additional insight into the ability of TSN to protect muscles from BoNT-induced paralysis.

2   Methods

2.1   Muscle tensions

Experiments were performed *in vitro* on hemidiaphragms dissected from male CD-1 mice (20–25 g). Mice were housed in Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC)- approved facilities with food and water available *ad libitum*. The animals were sacrificed by an overdose of isofluorane and decapitated. Hemidiaphragms with attached phrenic nerves were mounted in tissue baths at 37°C and immersed in an oxygenated physiological solution of the following composition (mM): NaCl, 135; KCl, 5.0; $MgCl_2$, 1.0; $CaCl_2$, 2.0; $NaHCO_3$, 15.0; Na_2HPO_4, 1.0 and glucose, 11.0. The solution was bubbled with a gas mixture of 95% O_2/5% CO_2 and had a pH of 7.3. The phrenic nerve was stimulated with 0.2-msec pulses at 0.03 Hz. The resulting muscle twitches were measured with WP FORT isometric force transducers (WP Instruments, Sarasota, FL), digitised and analysed offline with pClamp software (Molecular Devices, Sunnyvale, CA). Resting tension was adjusted to 1.0 g to obtain optimal nerve-evoked tensions. All drug and toxin concentrations were expressed as final concentration in a 10-ml tissue bath. Muscles were equilibrated for 20 min prior to the first recording and for 10 min after each drug or solution change prior to recording of muscle tensions.

2.2   Drug and toxin preparation

TSN ($C_{30}H_{38}O_{11}$; MW = 574; Figure 1) was obtained under a Cooperative Research and Development Agreement (CRADA) with the Department of Pharmacology and Toxicology, SUNY at Buffalo and was 98% pure. Additional samples of TSN with comparable purity were obtained as a gift from Dr. Kim Janda (Scripps Research Institute, San Diego, CA). TSN was dissolved in dimethylsulphoxide (DMSO) at a concentration of 1 mM and added to the physiological solution in the muscle bath at 0.1–10 μM.

BoNT/A, /B and /E were obtained from Metabiologics (Madison, WI) in their complex forms. Stock solutions of BoNT were prepared in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) and maintained at −30°C. BoNT/B and BoNT/E were activated (nicked) with trypsin type XI at pH 6 and then treated with an excess of soybean trypsin inhibitor prior to use.

2.3   Data analysis

Data are expressed as fraction of the control twitch tensions obtained before the addition of toxin. TSN- and BoNT-treated diaphragms were compared by assessing the 50% muscle paralysis time. This was defined as the time from toxin addition to the time that tensions declined to 50% of their initial value. Unless stated otherwise, data are reported as mean ± SE from 3–6 muscles. Statistical analysis was performed by a one-way ANOVA followed by Bonferroni's multiple comparisons post-tests or by an unpaired *t* test, as appropriate (Graphpad, Prism, San Diego, CA). *P* < 0.05 was considered to be statistically significant.

2.4   Molecular modelling

TSN was initially minimised in vacuum using Insight/Discover software (Accelrys, Inc., San Diego, CA, USA). TSN was then manually docked into a previously constructed BoNT/A pore model (Sheridan et al., 1997). One potential binding site was discovered. Close contacts of the docked molecule were relieved by constrained minimisation. This consisted of 300 steps at 1 fs per step using Constant Valence Force Field (CVFF) with tethered protein backbone atoms (quadratic tether, tether parameters = 1000.0 and 1000.0). An energy minimum was not reached during the simulations. The total movement of the protein backbone during minimisation was insignificant.

3   Results

3.1   Effect of TSN on muscle tension

To determine appropriate working concentrations of TSN for antagonism of BoNT, muscle tensions were recorded in the presence of 1, 3 and 10 µM TSN. Addition of 1 or 3 µM TSN had no significant effect on neurally elicited muscle tensions during the 3-h period of observation (Figure 2). The small reduction in tension from initial values can be accounted for by the decline in viability of the *in vitro* preparation over the 3-h time course of recording; reductions of this magnitude were also observed in control muscle (data not shown). When the concentration of TSN was increased to 10 µM, there was a rapid decline of muscle tension, falling to 55 and 8% of initial values after 30 and 60 min of TSN addition, respectively. Because of the inhibitory actions of TSN at 10 µM, subsequent experiments were carried out with concentrations of TSN ≤ 3 µM.

Figure 2   Twitch tensions following supramaximal stimulation of the phrenic nerve in the presence of 1–10 µM TSN. Values are plotted as a fraction of twitch tension prior to drug addition. Tensions that differ significantly from control ($P < 0.05$) are indicated by asterisks. DMSO, the vehicle for TSN, had no effect on muscle tension at 1%, the highest concentration used in this study (data not shown)
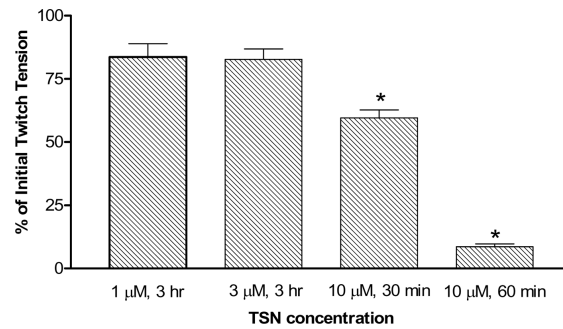
3.2   Concentration-response characteristics of TSN pre-treatment in antagonising BoNT/A

Figure 3 shows the effect of TSN concentrations ranging from 0.1 µM to 3 µM in antagonising the actions of BoNT/A on muscle tension. In the presence of 20 pM BoNT/A, muscle tensions declined rapidly after a brief latent period, culminating in complete muscle paralysis in approximately 2 h (half-time = 45.2 ± 4.4 min). A 30-min pre-treatment by 0.1 µM TSN produced no alteration in the paralytic time relative to that observed in the presence of BoNT/A alone (Figure 3). Raising the concentration of TSN to 0.3 µM led to a small increase in the time to 50% paralysis, which was not statistically significant. Increasing the TSN concentration to 1 and 3 µM, however, led to significant delays in the BoNT/A-mediated time-to-block of 2.5- and 3.2-fold, respectively ($P < 0.05$). The effect of 1 and 3 µM TSN on the BoNT/A paralytic time is noteworthy since it exceeds that produced by most BoNT antagonists examined to date (Adler et al., 2007).

Figure 3   Effect of a 30-min pretreatment by 0.1, 0.3, 1 or 3 µM TSN on the time to 50% paralysis in isolated mouse diaphragm muscles exposed to 20 pM BoNT/A. Significant differences ($P < 0.05$) are denoted by asterisks (see online version for colours)
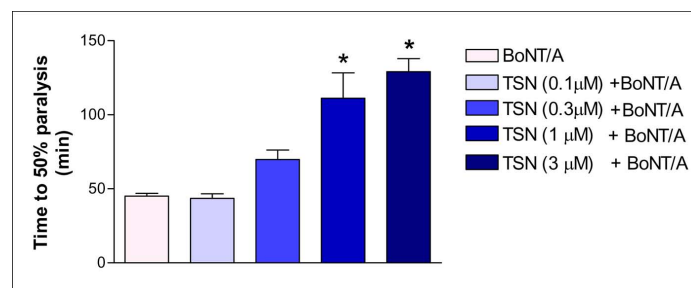
3.3 Effect of simultaneous and post-exposure of TSN on BoNT/A-mediated muscle paralysis

As illustrated in Figure 4, TSN (3 µM) also antagonised BoNT/A-mediated muscle paralysis when applied simultaneously with the toxin ($P < 0.05$). The protection observed after simultaneous addition of TSN and BoNT/A was similar to that observed with a 30-min pre-treatment of TSN, resulting in a 3.3-fold increase in the time to 50% paralysis for the former (Figure 4). If TSN was added 30 min after BoNT/A, however, no protection in toxin-mediated muscle paralysis was observed (Figure 4), even though a substantial fraction of the initial tension remained at the time of TSN addition ($66.5 \pm 7.1\%$ of initial tension, range 57–82%, $n = 5$). The brief window for intervention provided by TSN against BoNT is similar to that observed with proton pump inhibitors and antimalarial agents (Sheridan, 1996; Deshpande et al., 1997; Sheridan et al., 1997).

Figure 4 TSN (3 µM) delays the time to 50% paralysis of isolated mouse diaphragm muscle when added simultaneously with 20 pM BoNT/A, but not when added 30 min after BoNT/A. Asterisk indicates significant difference from 50% paralysis time observed in the presence of BoNT/A alone ($P < 0.05$) (see online version for colours)
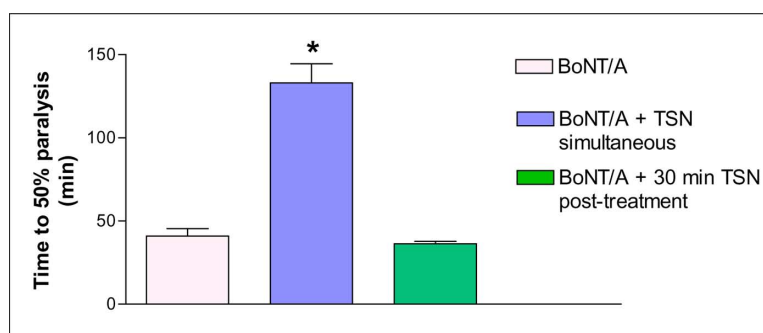
3.4 Effect of TSN on muscle paralysis produced by BoNT/B or /E

On the basis of results obtained with TSN on BoNT/A-mediated muscle paralysis, it was of interest to determine the effect of TSN on paralysis produced by BoNT/B or /E. Owing to the lower relative potencies of BoNT/B or /E, higher concentrations of these serotypes were used to obtain a similar time to block as that observed with 20 pM BoNT/A. For BoNT/B, 100 pM produced a comparable time-to-block (Figure 5). A 30-min pre-treatment with 1 µM TSN prior to exposure of muscles to 100 pM BoNT/B resulted in a 2.2-fold increase in the time to reach 50% muscle paralysis ($P < 0.05$). For BoNT/E, 200 pM was used to obtain the desired time-to-block and pre-treatment by 1 µM TSN produced a 2.1-fold increase in the time to reach 50% paralysis ($P < 0.05$).

Figure 5   Pretreatment for 30 min with 1 µM TSN delays the time to 50% paralysis of isolated
                 mouse diaphragm muscle exposed to 100 pM BoNT/B or 200 pM BoNT/E.
                 The differences in paralysis times between TSN-pretreated muscles and those exposed
                 to BoNT/B or /E alone were significant ($P < 0.05$) as indicated by asterisks (see online
                 version for colours)
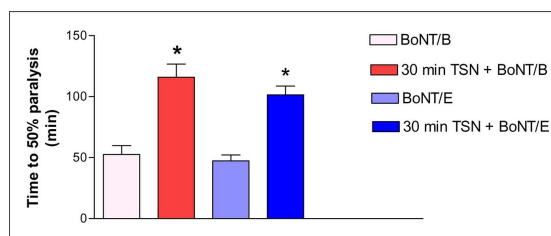
3.5   TSN interactions with the BoNT/A translocation pore

Among the possible mechanisms for the protective action of TSN is inhibition of the
translocation channel responsible for delivering the catalytic light chain of BoNT from
endosome to cytosol (Koriazova and Montal, 2003). To test this hypothesis, a molecular
model of the translocation channel was generated from coordinates provided by
Dr. M. Montal (University of California, San Diego) and TSN was docked to the outer
surface of the channel (Figure 6). TSN (ball and stick model) was found to bind tightly
with the open conformation of the BoNT channel leading to its occlusion (Figure 6). This
occlusion would be expected to impede the translocation of the light chain across the
endosomal membrane (Simpson, 2004) and slow the development of intoxication.
Blockade of the channel is not likely to prevent intoxication, since an equilibrium would
be expected between the open and occluded channel such that opportunities would still be
available for entry of the light chain (Sheridan et al., 1997; Koriazova and Montal, 2003).

Figure 6   Model of TSN interacting with the BoNT translocation pore. Panel A shows a ball and
                 stick figure of vacuum minimised TSN docked into a previously constructed model of
                 the BoNT/A pore (Sheridan et al., 1997). The helical backbone of the BoNT/A pore is
                 displayed in green with yellow indicating charged regions of the pore. Panel B shows
                 a ball and stick model of TSN docked into the BoNT pore after limited minimisation as
                 described in the methods. The BoNT pore is shown with a quick solvent accessibility
                 surface. The absence of close contacts between TSN and pore leads us to predict that
                 the binding is predominantly steric. One hydrogen bond but no charge-charge
                 interactions were found in the minimised structure (see online version for colours)
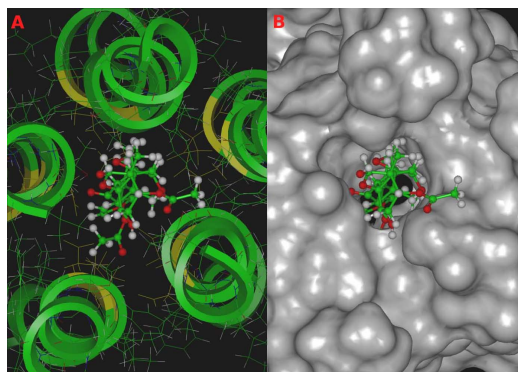
4   Discussion

The results of this investigation demonstrate that TSN antagonises the action of BoNT on mouse diaphragm muscle, making TSN a potential candidate for development as a BoNT therapeutic (Adler et al., 2007). Positive attributes of TSN included its ability to produce up to a 3-fold slowing in the time to 50% paralysis in the mouse diaphragm muscle (Figures 3–5) and its efficacy against the 3 serotypes of BoNT that are responsible for most human intoxications (BoNT/A, /B and /E). The 3-fold slowing in the BoNT-mediated paralysis compares favourably with the most effective BoNT antagonists reported to date, including the zinc chelator TPEN (Adler et al., 1997; Simpson et al., 2001), the antimalarial agents choloroquine, quinacrine and amodiaquine (Deshpande et al., 1997; Sheridan et al., 1997) and the ganglioside binding antagonist *Triticum vulgaris* lectin (Bakry et al., 1991; Adler et al., 2007). In addition, TSN has an extensive history of human use in China and appears to be well tolerated (Tada et al., 1999; Shi and Wang, 2004).

In spite of these encouraging findings, TSN was found to have a number of limitations. For example, although TSN was effective in antagonising BoNT at concentrations up to 3 µM (Figures 3–5), a half-log increase in concentration to 10 µM led to nearly complete muscle paralysis in 60 min (Figure 2). In addition, TSN was ineffective in antagonising BoNT when given 30 min after toxin exposure (Figure 4), even though a substantial fraction of the initial tension was still present. Although BoNT/A was the only serotype tested after the onset of muscle paralysis, it is reasonable to assume that the finding would apply to serotypes B and E as well.

The mechanism of action of TSN is complex (Shi and Wang, 2004). Single channel patch-clamp recordings have revealed that TSN blocks both large conductance calcium-activated potassium channels (Wang and Shi, 2001) and delayed rectifier potassium channels (Hu et al., 1997). In addition, TSN has been shown to enhance pre-synaptic calcium currents in mouse triangularis sterni muscle at low concentrations (1–1.7 µM), but to inhibit these currents at higher concentrations (≥17 µM) (Ding et al., 2001). Inhibition of nerve terminal calcium currents may be responsible for the reduction in muscle tension observed with 10 µM TSN in this study (Figure 2).

The beneficial effect of TSN was suggested to result from inhibition of potassium channels and a consequent increase in calcium influx leading to an enhanced release of ACh (Shi and Wang, 2004). Blockade of potassium channels is unlikely to be the mechanism underlying BoNT antagonism: drugs that act in this manner are able to reverse muscle paralysis caused by BoNT/A more effectively than that produced by other serotypes and they are effective both before and after BoNT challenge (Simpson, 1986; Adler et al., 1995, 1996, 2000). TSN was unable to reverse muscle paralysis when added after BoNT and was equally effective against serotypes A, B and E.

The ability of TSN to antagonise multiple BoNT serotypes implies action at a common step in the intoxication process. The most likely candidate is toxin translocation, since inhibition at this step could account for the absence of serotype selectivity and the lack of efficacy after onset of paralysis (Simpson, 2004; Adler et al., 2007). On the basis of docking studies with the BoNT translocation channel (Figure 6), it is suggested that

TSN acts by occluding the BoNT channel to slow the rate of toxin translocation. Although this mechanism is consistent with the results of this study, it would not explain the findings of Jing et al. (1985) in which TSN administered to rhesus monkeys 24 h after BoNT/A was highly protective. It is possible that the protection observed in these animals was due to the action of a metabolite and not of the parent compound.

BoNT has proven to be a difficult target for pharmacological intervention. Most compounds that exhibit efficacy in cell-free enzymatic assays have been found to have little or no efficacy in isolated nerve-muscle preparations and are unable to protect animals *in vivo* (Adler et al., 1997, 2007; Dickerson and Janda, 2006). Consequently, the results with TSN, while of limited promise, are still encouraging and a study with analogues of TSN may be profitable to pursue.

Acknowledegments

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